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(54) Title: CATIONIC AMPHIPHILES

(57) Abstract

Cationic amphiphiles are provided that are alkyl or alkoxyalkyl O-phosphate esters of diacylphosphatidyl zwitterionic compounds such as phosphatidylcholine or phosphatidyl ethanolamine. The amphiphiles can be used as carriers for delivering macromolecules intracellularly.

84:7413-7417); mRNA (Malone and Keloff, *Proc. Nat'l. Acad. Sci. USA* (1989) 86:6077-6081); and purified transcription factors (Debs *et al., J. Biol. Chem.* (1990) 265: 10189-10192) in functional form.

SUMMARY OF THE INVENTION

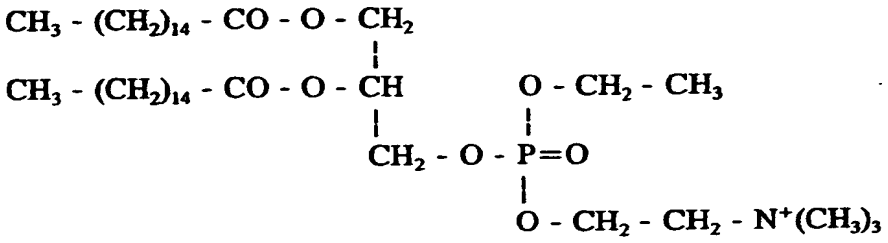
Biodegradable cationic amphiphiles are provided together with methods for their use. The amphiphiles are prepared from naturally occurring, synthetic or semisynthetic phosphoglycerides by modification of the phosphate moiety of the phosphoglyceride with a neutral group. The cationic amphiphiles are capable of forming complexes with nucleic acids, and other biological compounds and the nucleic acid complexes are capable of transforming mammalian cells. The amphiphiles of the invention yield non-toxic degradation products when subjected to endogenous enzymatic processes.

DESCRIPTION OF SPECIFIC EMBODIMENTS

Metabolizable cationic amphiphilic materials are provided which are useful as carriers for biologically active molecules, such as antibiotics or nucleic acids used in cell transformation processes. The use of the cationic amphiphiles as nucleic acid carriers is described in detail, since the compositions prepared using the amphiphiles are particularly efficacious for this purpose. However, the amphiphiles are also useful in standard drug delivery regimens, such as for the delivery of antibiotics to the lungs of a patient. In particular, complexes of the amphiphiles with DNA (for the transformation of cells in mammalian tissues) give rise to reduced amounts of toxic cleavage products when subject to the metabolic degradation process.

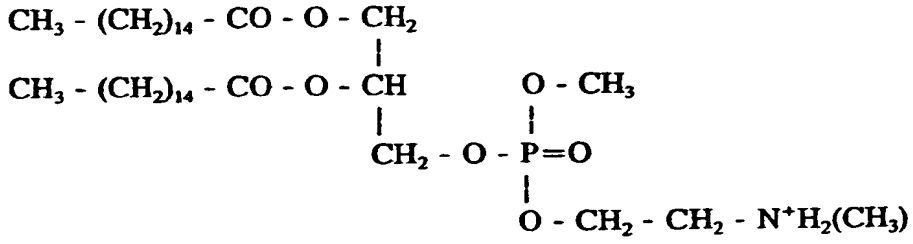
The invention in particular is directed to phosphorus-containing cationic amphiphiles which are nontoxic themselves and which yield by-products, such as those produced by enzymatic cleavage, which are nontoxic to a host organism or which are identical to substances endogenous to a host organism. These amphiphiles thus offer the advantage that they can readily be used in humans, since they can be used repeatedly without the accumulation of toxic by-products.

apparent from the above formula I and the definitions of the terms as provided. In general, the cationic amphiphiles are O,O'-esters of a diacylphosphatidyl acid where X and Z are the esterifying groups. By analogy to the conventional nomenclature for the materials of formula III, the X group is designated in terms of the hydroxylic compound from which it is derived. Thus, in cations wherein X is cholinyl, i.e., $-CH_2-CH_2-N^+(CH_3)_3$, the cations are O-alkyl or O-alkoxyalkyl esters of a diacylphosphatidylcholine. In similar manner, an O-ester of a diacylphosphatidyl acid derivative in which X is $-CH_2-CH_2-NH_2$ is referred to as a 0-alkyl ester of a diacylphosphatidylethanolamine. By way of specific illustration, the cationic amphiphile of the formula



is O-ethyl dipalmitoylphosphatidylcholine.

The cationic amphiphile of the formula



is a methyl quaternary ammonium derivative of O-methyl dipalmitoylphosphatidyl-ethanolamine.

Cationic amphiphiles of formula I are produced by conventional synthetic processes. For example, a zwitterionic diacylphosphatidyl acid, e.g., a diacylphosphatidylcholine, is esterified by a substantially equimolar quantity of the hydroxylic compound from which Z is derived, e.g., methanol. In practice, esterification is facilitated by the presence of a sulfonyl halide such as

cationic lipids can be used alone or combined with other lipids in formulations for the preparation of lipid vesicles or liposomes for use in intracellular delivery systems. Uses contemplated for the lipids of the invention include transfection procedures corresponding to those presently known that use amphiphilic lipids, including those using commercial cationic lipid preparations, such as Lipofectin ™, and various other published techniques using conventional cationic lipid technology and methods. The cationic lipids of the invention can be used in pharmaceutical formulations to deliver therapeutic agents by various routes and to various sites in an animal body to achieve a desired therapeutic affect.

Because such techniques are generally known in the art, background information and basic techniques for the preparation of pharmaceutical compositions containing lipids will not be repeated at this time. A reader unfamiliar with this background information is referred to the publications under the heading Relevant Literature above and further to U.S. Patent No. 5,264,618. This last-cited patent describes a number of therapeutic formulations and methods in detail, including examples of the use of specific cationic lipids (different from those described here) that can be followed in detail by substituting the cationic lipids of the present invention for those described in the patent. Compositions of the present invention will minimally be useable in the manner described in the patent, although operating parameters may need to be modified in order to achieve optimum results, using the specific information provided for compounds of the invention in this specification along with the knowledge of a person skilled in the arts of lipid preparation and use.

The lipids of the present invention have been shown to be particularly useful and advantageous in the transfection of animal cells by genetic material. Additionally, since these compositions are degraded by enzymatic reactions in animal cells to components that are typically indigenous to the cells, the compositions provide a number of advantages in the area of low toxicity when compared to previously known cationic lipids. These and other advantages of the invention are discussed in detail below. The remainder of this discussion is directed principally to selection, production, and use parameters for the cationic

mixed in solutions which do not themselves induce aggregation of the DNA:lipid carrier complexes, such as sterile water. The expression cassette (DNA) is mixed together with each of the lipid carriers to be tested in multiple different ratios, ranging as an example from 4:1 to 1:10 (micrograms of DNA to nanomoles of cationic lipid or total lipid, if a lipid mixture is present). Examination of the stability of the resulting mixtures provides information concerning which ratios result in aggregation of the DNA:lipid carrier complexes and are therefore not useful for use in vivo, and which complexes remain in a form suitable for aerosolization. The ratios which do not result in aggregation are tested in animal models to determine which of the DNA:lipid carrier ratios confer the highest level of transgene expression in vivo. For example, for aerosol-based transfection, the optimal DNA:lipid carrier ratios for lipid mixtures such as N-[1-(2,3-dioleyloxy)-propyl]-N,N,N-triethylammonium chloride(DOTMA):dioleoylphosphatidylethanol-amine(DOPE) (the components of this mixture being present in a 1:1 weight ratio) and dimethyl dioctadecyl ammonium bromide (DDAB):Chol (1:1) are 1 to 1. For O-ethyl egg phosphatidylcholine (E-EPC) or especially O-ethyl dimyristoyl-phosphatidylcholine (E-DMPC) in a 1:1 weight ratio with cholesterol, the DNA:lipid carrier ratio is preferably in the range of from 1.5:1 to 2:1.

If the cationic amphiphile is used for injection, then it need be evaluated only for whether it is effective for transfection of a target cell.

Particular cells can be targeted by the use of particular cationic lipids for preparation of the lipid-mixture carriers, for example, by the use of E-DMPC to target lung cells preferentially, or by modifying the amphiphiles to direct them to particular types of cells using site-directing molecules. Thus antibodies or ligands for particular receptors may be employed, to target a cell associated with a particular surface protein. A particular ligand or antibody can be conjugated to the cationic amphiphile in accordance with conventional techniques, either by conjugating the sitedirecting molecule to a lipid for incorporation into the lipid bilayer or by providing a linking group on a lipid present in the bilayer for linking to a functionality of the site-directing compound. Such techniques are well known to those skilled in the art.

complex due to binding of the negatively charged DNA to the cationic lipid carriers. SUVs find use with small nucleic acid fragments as well as with large regions of DNA (≥250kb).

In preparing the lipid carrier-nucleic acid complex for nebulization, care should be taken to exclude any compounds from the mixing solution which promote the formation of aggregates of the lipid carrier-nucleic acid complexes. Large particles generally will not be aerosolized by the nebulizer, and even if aerosolized would be too large to penetrate beyond the large airways. Aggregation of the lipid carrier-nucleic acid complex is prevented by controlling the ratio of DNA to lipid carrier, minimizing the overall concentration of DNA:lipid carrier complex in solution, usually less than 5 mg DNA/8 ml solution, and avoiding the use of chelating agents such as EDTA and/or significant amounts of salt, either of which tends to promote macro-aggregation. The preferred excipient is water, dextrose/water or another solution having low or zero ionic strength. Further, the volume should be adjusted to the minimum necessary for deposition in the lungs of the host mammal, while at the same time taking care not to make the solution too concentrated so that aggregates form. Increasing the volume of the solution is to be avoided if possible due to the need to increase the inhalation time for the host animal to accommodate the increased volume. In some cases, it may be preferable to lyophilize the lipid carrier-nucleic acid complexes for inhalation. Such materials are prepared as complexes as described above, except that a cryoprotectant such as mannitol or trehalose is included in the buffer solution which is used for preparation of the lipid carrier-DNA complexes. Any glucose generally included in such a buffer is preferably omitted. The lipid carrier complex is rapidly freeze-dried following mixing of the lipid and DNA. The mixture can be reconstituted with sterile water to yield a composition which is ready for administration to a host animal.

Where the amphiphiles form liposomes, the liposomes may be sized in accordance with conventional techniques, depending upon the desired size. In some instances, a large liposome injected into the bloodstream of an animal has higher affinity for lung cells as compared to liver cells. Therefore, the particular size range may be evaluated in accordance with any intended target tissue by

placed in a 100 ml round bottom flask in chloroform solution, and the chloroform was removed by evaporation. To the lipid film was added 6 ml of dry N,N-dimethylformamide, 3 ml of dry methanol, and 2.5 ml of dry lutidine. The lipid dissolved readily in the solvent mixture. p-Toluenesulfonyl chloride (1.2 g) was added, which dissolved readily. The mixture was allowed to react for 1 hour at room temperature. The flask was then chilled on ice, and 1 ml of distilled water was added. After 15 minutes, the mixture was transferred to a 1 liter flask, together with 20 ml of ethanol. The solvent was removed by rotary evaporation. The resultant residue was dissolved in 30 ml of chloroform, to which was added 30 ml of methanol and 30 ml of distilled water.

After vigorous shaking, the flask was allowed to stand until the contents separated into a lower chloroform layer and an upper methanol/water layer. The chloroform layer was removed and transferred to a fresh flask. To this solution was added a further 30 ml of methanol and 30 ml of water. Three gm of NaCl was dissolved in the water to aid separation of the phases. The mixture was shaken vigorously, allowed to stand, and the chloroform layer was transferred once more to a fresh flask. 30 ml of methanol and 30 ml of water together with 3 gm of NaCl was added again, the mixture was shaken, and the chloroform layer was removed. The washed phospholipid was evaporated to give a yellow oil, which was dissolved in 15 ml of chloroform. This solution was then applied to a 1.5 x 10 cm column of silica gel in chloroform. After the sample had been loaded onto the column, the column was washed with 100 ml of chloroform, the eluant being discarded. The solvent was then changed to a mixture of chloroform, methanol, water, and glacial acetic acid, in the proportions 69:27:2.3:1.5 by volume. This mixture will subsequently be referred to as solvent A. The column was eluted with 100 ml of solvent A, and the eluant was collected in eight fractions. Thin layer chromatography of the fractions was carried out using solvent A, and the presence of various compounds was detected first by exposing the plates to iodine vapor, and second by spraying the plates with a phosphate spray. In this solvent system, the original phospholipid had an Rf value of approximately 0.2. Fractions 1-3 contained a single compound with an Rf value of approximately 0.5. Fractions 4-6 appeared to contain some residual

proceed for 2 hours before the addition of water. Reaction with propanol and butanol was allowed to proceed for 24 hours, and the reaction appeared only 70% complete from TLC analysis.

Example 3: Synthesis of Modified Phosphatidylethanolamines

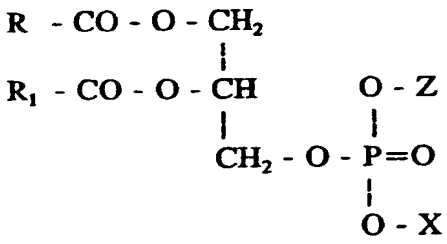
In addition to the methods described above for phosphatidylcholine, a similar approach can be adopted to the modification of several other naturally occurring phospholipids to create degradable cationic amphiphiles. These include phosphatidylethanolamine. For phosphatidylethanolamine, the procedure of Example 1 is followed except that it is preferred to protect the primary amino group of the lipid prior to synthesis, and then to remove the protecting group afterwards.

In accordance with the subject invention, compositions and methods are provided for delivering drugs to a cell. The method minimizes non-specific interaction of the drug with cells other than the targeted cells. By employing weakly acidic drugs, which are substantially impermeable to lipid bilayers, leakage of the drug from the amphiphiles is minimized, so as to minimize non-specific effects of the drug. Furthermore, the drug is efficiently incorporated into the cytosol of the cell, where it will be most effective.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.

All publications and patent applications cited in this specification are herein incorporated by reference to the same extent as if each individual application or publication was specifically and individually incorporated by reference.

6. A cationic amphiphile of the formula



wherein Z is alkyl or alkoxyalkyl,

R and R_1 are independently straight-chain, aliphatic hydrocarbyl groups of from 11 to 29 carbon atoms inclusive and

X is a cationic moiety of the formula

$-CH_2-(CH_2)_n-N^+(R_2)_3$ wherein n is an integer from 1 to 4 inclusive and R_2 independently is hydrogen or lower alkyl with at least one R_2 being hydrogen.

7. The amphiphile of Claim 6 wherein n is 1.

8. The amphiphile of Claim 7 wherein two R_2 moieties are hydrogen.

9. The amphiphile of Claim 8 wherein R_2 is hydrogen or methyl.

10. The amphiphile of Claim 7 wherein Z is methyl or ethyl.

11. The cationic amphiphile according to Claim 1, wherein said composition is lyophilized or is in a sonicated suspension or is mixed with cholesterol.

12. The cationic amphiphile according to Claim 11, wherein an antibody is bound to said cationic amphiphilie.

INTERNATIONAL SEARCH REPORT

Int...ational application No.
PCT/US94/07071

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C07F 9/02; C07H 21/00; A61K 35/00; C07 J 11/00
US CL :554/80; 552/544; 424/114; 536/22.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 554/80; 552/544; 424/114; 536/22.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CAS ONLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Journal of Organic Chemistry, volume 51, no. 12, issued 13 June 1986, Karol S. Bruzik et al, "A general method for the synthesis of glycerophospholipids", pages 2368-2370, see pg. 2369, compound 5.	1-15

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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